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Evaluation of phenotypic and genotypic methods for coagulase typing of MRSA isolates from diverse sources

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Abstract
Methicillin-resistant *Staphylococcus aureus* (MRSA) is a significant pathogen in both human and veterinary medicine due to its resistance to multiple antibiotics and its zoonotic potential. This study aimed to compare the phenotypic detection of coagulase using the tube coagulase test (TCT) with plasma from humans and seven animal species, against genotypic detection through *coa* gene PCR among MRSA isolates from various sources. A total of 203 samples were collected from human pus, animal pus, mastitis milk, and unprocessed meat in Bikaner, Rajasthan, out of which 78 isolates were confirmed as MRSA based on 23S rRNA and *mec*A gene amplification. TCT results showed that 75 (96.15%) isolates were coagulase-positive at 5 hours, and one additional isolate turned positive at 24 hours. Human plasma showed the strongest and earliest coagulation, followed by plasma from camel, buffalo, horse, sheep, goat, cattle, and chicken. Coagulation strength was often higher when the plasma origin matched the host species of the isolate. All 78 isolates tested positive for the *coa* gene by PCR, showing greater sensitivity than TCT. Amplicon sizes varied from 350 bp to 850 bp, with five distinct profiles: 700 bp (38.46%), 600 bp (33.33%), 850 bp (20.51%), 350 bp (3.85%), and dual bands (600 and 850 bp) in 3.85% of isolates, with the dual bands observed only in animal-origin isolates. Among human-derived isolates, 700 bp and 850 bp genotypes were equally dominant, while 700 bp was most frequent in meat samples. The findings highlighted the limitations of TCT, particularly with animal isolates, and emphasized the value of *coa* gene PCR for accurate detection and genotypic characterization of MRSA.

Keywords: MRSA, *S. aureus*, coagulase gene, tube coagulase test, polymorphism

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) remains a critical pathogen in both human and veterinary healthcare, contributing to a wide array of infections and exhibiting resistance to multiple antibiotics (Turner *et al.*, 2019) [31]. Accurate identification and characterization of MRSA isolates are essential for effective treatment and infection control strategies (Geetha *et al.*, 2015) [13]. Among its virulence factors, coagulase, an enzyme responsible for converting fibrinogen to fibrin, is a traditional and significant marker used to distinguish *S. aureus* from coagulase-negative staphylococci (CNS) (Locatelli *et al.*, 2023) [19]. The tube coagulase test (TCT) remains a standard phenotypic assay for detecting coagulase activity (Moustafa *et al.*, 2021) [23]. Despite its simplicity and low cost, the test is influenced by variables such as the source of plasma, strain-specific coagulase production, and incubation duration (Javid *et al.*, 2018) [17]. Instances of false-negative or delayed coagulase responses have been documented, particularly among animal-origin isolates, raising concerns regarding the reliability of TCT as a standalone diagnostic method (Zamani *et al.*, 2023) [36].

At the molecular level, the *coa* gene, which encodes the coagulase protein, has emerged as a reliable target for genotypic detection and epidemiological typing of *S. aureus* (Mahmoudi *et al.*, 2017) [21]. Polymorphisms in the 3' region of the gene, particularly due to variable tandem repeats, have been widely used for strain differentiation (Dallal *et al.*, 2016). PCR-based *coa* typing provides higher sensitivity and specificity compared to phenotypic methods and is particularly useful in identifying atypical or weakly coagulase-producing strains

(Andrade-Figueiredo *et al.*, 2023) [2]. Recent studies have revealed discrepancies between phenotypic coagulase expression and the presence of the *coa* gene, suggesting potential mutations or regulatory mechanisms that influence protein expression. For instance, Locatelli *et al.* (2023) [19] reported that a single-nucleotide deletion in the *coa* gene led to persistent coagulase-negative expression despite the presence of *S. aureus*, indicating the importance of incorporating molecular methods for accurate identification. Given the increasing recognition of host-adapted MRSA strains and the variability of diagnostic outcomes depending on the isolate source, a comparative analysis of TCT and *coa* gene detection becomes crucial. This study aims to evaluate the concordance between phenotypic and genotypic detection of coagulase among MRSA isolates from diverse origins and to assess the influence of plasma source and incubation time on the performance of the TCT.

Materials and Methods

Sample Collection and Identification

The present study included a total of 203 samples collected from various human and animal sources in and around Bikaner, Rajasthan (India). A total of 45 human pus samples were collected from PBM Hospital, Bikaner. Animal pus samples (n = 60) were obtained from different species, including dogs (15), cattle (15), camels (15), and horses (15), at the Teaching Veterinary Clinical Complex (TVCC), College of Veterinary and Animal Science (CVAS), Bikaner. Additionally, 38 mastitis milk samples were collected from bovines presented at TVCC, CVAS, Bikaner, comprising 19 samples from cattle and 19 from buffaloes. Lastly, 60 unprocessed meat samples were included, consisting of 30 poultry meat swabs and 30 goat meat swabs collected from local meat shops in Bikaner. The samples were collected in sterile HiMedia hiculture collecting device and immediately transferred to the laboratory on ice for further processing.

Isolation and identification of MRSA

Cowan and Steel (1975) [9] and Quinn *et al.* (1994) [27] methods were used for the isolation and identification of *S. aureus*. Each sample was swabbed on to the nutrient agar plate and then incubated overnight at 37 °C. Next day different bacterial colonies were closely observed for their morphology, colour and consistency. For primary identification of *S. aureus* Gram's staining was used. For reconfirmation, the cultures were streaked on Mannitol salt agar, Baird Parker agar (Himedia Laboratories) and MeReSa agar. The colonies were further confirmed using the biochemical identification done by VITEK® 2 automated system (bioMerieux, France). The presumptively identified MRSA colonies were maintained at −80 °C in glycerol stocks for further analysis. Genomic DNA was extracted from a freshly grown culture in nutrient broth by using DNA was isolated by QIAamp DNA Mini Kit (Qiagen, USA). The extracted DNA was examined for purity and concentration using a NanoDrop spectrophotometer thereafter stored at −20 °C. The isolates were further genotypically confirmed by 23S rRNA species-specific polymerase chain reaction (PCR) (Straub *et al.*, 1999) [30] and methicillin resistance was confirmed by the presence of the *mec*A gene (Mehrotra *et al.*, 2000) [22].

Tube Coagulase Test

Blood was collected aseptically in anticoagulant-coated tubes and centrifuged at 2500 rpm for 15-20 min. The top layer of clear plasma was decanted into another sterilized test tube for further use in the test. The plasma was diluted to 1:10 in physiological saline solution and 0.5 ml of reconstituted plasma was taken in three test tubes. A 0.1 ml of an overnight test broth culture of MRSA was added to one tube and 0.1 ml of a broth culture of *S. epidermidis* was added to the second tube for negative control and another un-inoculated tube was kept as control. The tubes were rotated gently to mix the contents and incubated in a water bath at 37 °C. By slowly tilting the tube at a 90° angle, the tubes were examined at 1, 3, 5 and 24 hour compared with the control tubes. Clotting of plasma up to 5 hour was recorded as positive for "free" coagulase enzyme.

Amplification of *coa* gene

The *coa* gene amplification was done as per the method described by Hookey *et al.* (1998) [15] using F-5'-ATAGAGATGCTGGTACAGG-3' and R-5'-GCTTCCGATTGTTCGATGC-3' primers. PCR was performed in 0.2 ml thin-walled PCR tubes. A 25 µL PCR reaction mixture consisting of 12.5 µL of 2X DreamTaq Green PCR Master Mix (Thermoscientific, Mumbai, India), 0.5 µM of each primer, and 1 µL of template DNA was prepared and performed in a Veriti Thermal Cycler (Applied Biosystem). The following program was used for PCR cycles: 2 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 57 °C, and 1 min at 70 °C with a final extension of 5 min at 72 °C. Amplified products were separated by electrophoresis in a 1.2% agarose gel in 1X TBE buffer at a constant voltage of 4 V/cm, stained using ethidium bromide (0.5µg/ml) and 100 bp DNA ladder was used as a molecular marker. The gel was then visualized under a gel documentation system (ENDURO GDS).

Results

Tube coagulase test

Coagulase production by 78 MRSA isolates was assessed using the tube coagulase test (TCT) with plasma from eight animal species and humans. Among these, 75 isolates (96.15%) were coagulase-positive at the 5-hour reading, while 3 isolates (3.85%) were initially negative (1 from camel pus, 2 from goat meat) (Figure 1). Notably, one of the initially negative isolates from goat meat showed a delayed positive reaction at 24 hours. The degree of clot formation varied between isolates and among plasmas of different species. Human plasma consistently showed the highest rate of positive coagulase reactions across all sources, followed by plasma from camel, buffalo, horse, sheep, goat, cattle, and chicken in descending order (Figure 2). Interestingly, coagulase reactions were more frequent and stronger when the plasma source matched the host species of the isolate.

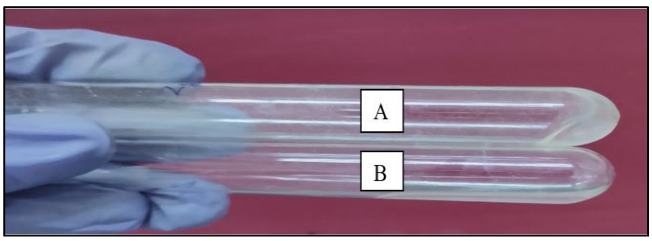

Fig 1: Tube coagulase test: A-Positive; B-Negative

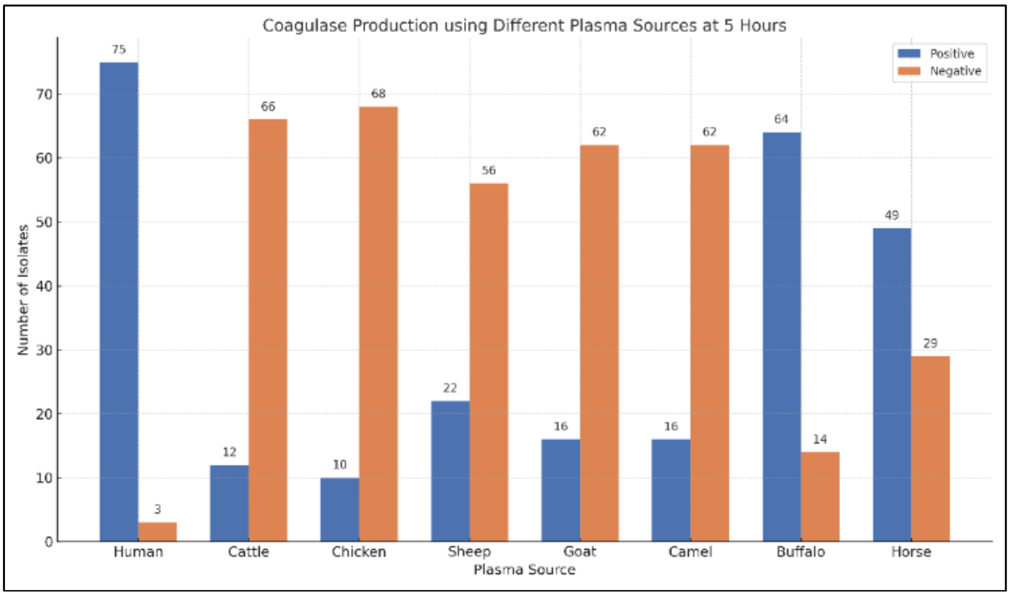

Fig 2: Comparison of coagulation of plasmas from different species of animals and human by MRSA isolates.

Amplification of *coa* gene:

All 78 MRSA isolates tested in this study were found to be positive for the *coa* gene by PCR, confirming the universal presence of the coagulase gene among the isolates. The PCR amplification revealed size polymorphism in the *coa* gene amplicons, ranging from 350 bp to 850 bp (Table 2, Figure 3). Based on these differences in amplicon size, five distinct genotypic patterns were identified among the isolates: 700 bp, 600 bp, 850 bp, 350 bp, and a combination of 600 bp and 850 bp (double bands) (Figure 4). The 700 bp amplicon was the most frequently observed pattern, present in 30 isolates (38.46%), followed by the 600 bp band in 26 isolates (33.33%), the 850 bp band in 16 isolates (20.51%), the 350 bp band in 3 isolates (3.85%), and double bands (600 bp and 850 bp) in 3 isolates (3.85%). Among the human-derived MRSA isolates, 700 bp and 850 bp genotypes were most commonly observed, each constituting 37.5% of the total. In animal-derived isolates, the 700 bp and 600 bp genotypes were predominant, appearing equally across various animal species including cattle, camel, goat, and horse. Notably, double-band profiles (600 bp and 850 bp) were observed exclusively in animal isolates, while absent in human samples. From mastitis milk samples, the 700 bp and 850 bp genotypes were the most prevalent, each accounting for 29.41% of the 17 isolates. In isolates from unprocessed meat samples (n = 20), the 700 bp genotype was dominant (45%), followed by the 600 bp genotype (40%) and the 350 bp band (10%).

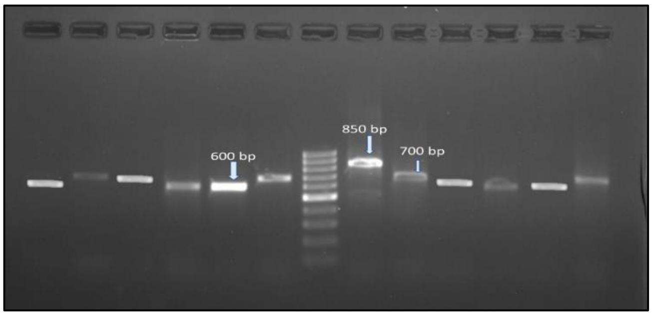

Fig 3: *coa* gene showing polymorphism with variable size amplicons

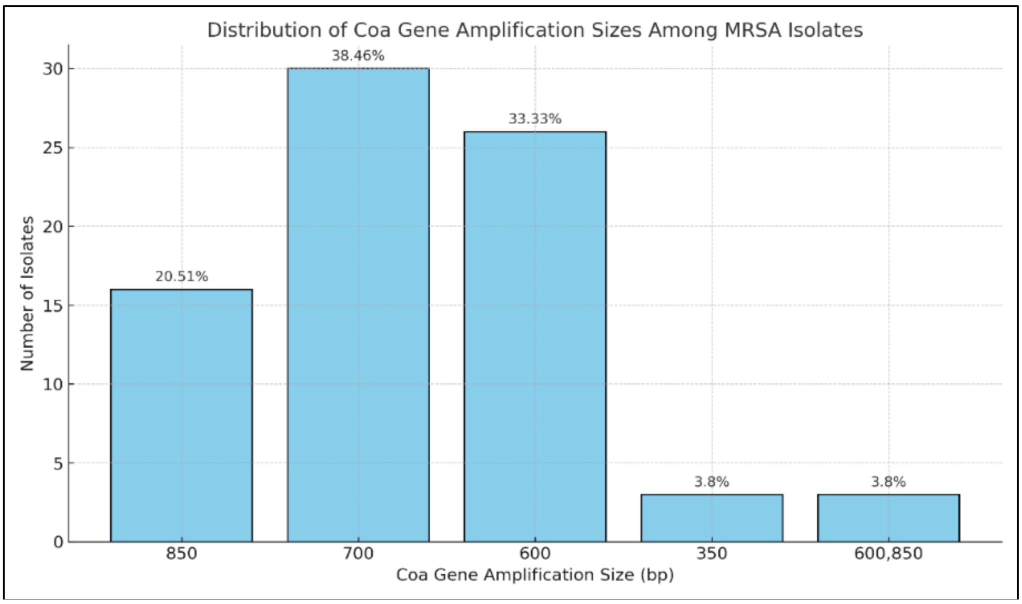

Fig 4: Distribution of *coa* gene among MRSA isolates irrespective of source.

Table 2: Distribution of *coa* gene among MRSA isolates from different sources

S. No.	Sample type	No. of samples	*coa* amplicon size	No. of isolates (%)
1.	Pus of human	16	850	6 (37.50)
			700	6 (37.50)
			600	4 (25.00)
2.	Pus of animals	25	850	4 (16.00)
			700	10 (40.00)
			600	10 (40.00)
			600,850	1 (4.00)
	Dog	6	850	1 (16.67)
			700	4 (66.67)
			600	1 (16.67)
	Cattle	7	850	1 (14.29)
			700	2 (28.57)
			600	4 (57.14)
	Camel	6	850	1 (16.67)
			700	2 (33.33)
			600	3 (50.00)
	Horse	6	850	1 (16.67)
			700	2 (33.33)
			600	2 (33.33)
			600,850	1 (16.67)
3.	Mastitis milk	17	850	5 (29.41)
			700	5 (29.41)
			600	4 (23.53)
			350	1 (5.88)
			600,850	2 (11.76)
	Cattle	9	850	4 (44.44)
			700	2 (22.22)
			600	1 (11.11)
			350	1 (11.11)
			600,850	1 (11.11)
	Buffalo	8	850	1 (12.50)
			700	3 (37.50)
			600	3 (37.50)
			600,850	1 (12.50)
4.	Unprocessed Meat	20	850	1 (5.00)
			700	9 (45.00)
			600	8 (40.00)
			350	2 (10.00)
	Chicken	7	850	1 (14.29)
			700	3 (42.86)
			600	3 (42.86)
	Goat	13	700	6 (46.15)
			600	5 (38.46)
			350	2 (15.38)

Discussion

Coagulase production, a key virulence attribute of *Staphylococcus aureus*, remains a widely used diagnostic criterion to differentiate *S. aureus* from coagulase-negative staphylococci (CoNS). In the present study, 10 out of 78 MRSA isolates exhibited coagulase positivity with plasma from all eight animal species and human sources, supporting the broad-spectrum coagulase activity of these isolates. However, the intensity and onset of clot formation varied considerably across different plasmas, echoing findings by Bhati *et al.* (2016) [6] and Sharma (2017) [29], who emphasized the differential coagulase response among plasma sources. Human plasma consistently demonstrated superior performance in detecting coagulase activity across most isolates, which is in agreement with findings of Yadav *et al.* (2015) [35] and Maddela *et al.* (2024) [20]. Consistent with this, Verma (2021) [33] found that 83.62% of MRSA isolates tested were positive in TCT. Nathiya *et al.* (2018) [24] also observed coagulase positivity in the majority of isolates except one that failed to coagulate human plasma, indicating occasional false negatives in phenotypic assays.

Interestingly, in the current study, a few isolates exhibited delayed coagulation-weak or no clot formation at 5 hours but positive reactions at 24 hours. Similar delayed reactions were documented by Boerlin *et al.* (2004) [7], who suggested prolonged incubation enhances sensitivity, especially in weakly coagulase-producing strains. Such findings underscore the role of incubation time in improving the diagnostic yield of the TCT. Moreover, false-negative TCT results may arise from low-level or non-expression of coagulase under *in vitro* conditions (Varettas *et al.*, 2005) [32], warranting molecular confirmation. Indeed, in the present study, three isolates negative by TCT were confirmed to carry the *coa* gene through PCR, highlighting the importance of integrating molecular diagnostics for definitive identification.

The phenomenon of coagulase-negative *S. aureus* has also been reported by Parth *et al.* (2016) [26], Bhagat *et al.* (2017) [3], Sharma *et al.* (2017) [29], and Yadav *et al.* (2018) [17], with varying frequencies, suggesting that phenotypic detection alone may not always reflect the genetic potential of isolates. Khichar and Kataria (2015) [18] found animal plasmas (particularly those from rabbit, buffalo, and cattle) to be more effective than human plasma in eliciting strong coagulation reactions. This discrepancy may stem from differences in strain-specific coagulase production, sample origin, or even handling and preparation of the plasma used.

The *coa* gene, encoding coagulase, is a key virulence marker and an important molecular target for the identification and differentiation of *Staphylococcus aureus* strains. In the present study, all 78 MRSA isolates demonstrated amplification of the *coa* gene, indicating 100% recovery, which aligns with the findings of Sharma (2017) [29], who also reported complete detection of the *coa* gene in *S. aureus* isolates. This consistency underscores the reliability of the *coa* gene as a molecular marker for *S. aureus*, particularly in methicillin-resistant strains. However, contrasting observations have been made by various researchers who reported the existence of *coa*-deficient strains in different proportions. Bhati *et al.* (2014) [5], Parth *et al.* (2016) [26], and Diwakar (2023) [12] reported variable frequencies of *coa*-negative isolates, which could be attributed to geographical variation, host species, or differences in sample types. Notably, Bhati (2019) [4] observed five distinct *coa* gene amplicons, suggesting genotypic diversity, which is in concordance with the current findings where multiple amplicon sizes were detected.

The predominant amplicon observed in the present study was 700 bp, a result that mirrors the findings of Sharma (2017) [29], who also recorded 700 bp as the most frequent band among isolates from various sources. In another related study, Hema *et al.* (2022) [14] identified six genotypic variants (Code I-VI) ranging from 300 bp to 800 bp among clinical isolates, with the 600 bp band being the most prevalent, supporting the existence of polymorphism within the *coa* gene.

Javid *et al.* (2018) [17], in contrast, reported that only 64.10% of their *S. aureus* isolates carried the *coa* gene and observed four distinct genotypes (514 bp, 595 bp, 757 bp, and 802 bp), indicating variability that differs from the current findings. Similarly, Sadiq *et al.* (2020) [28] found only 54%

positivity for the *coa* gene among MRSA isolates from meat shops in Pakistan, which is significantly lower than the present study, likely reflecting differences in strain ecology or regional practices. The observation of two distinct bands in some isolates aligns with findings from Dendani *et al.* (2016) [8], who speculated that dual bands might be due to the presence of mixed *S. aureus* strains or contamination during milking.

While the majority of current findings lie within a moderate amplicon size range, a wider diversity has been reported by other researchers, such as Dendani *et al.* (2016) [8] (300-980 bp) and Sharma *et al.* (2017) [29] (730-1130 bp). Deepak *et al.* (2024) showed 11 different types of amplicons, including 420 bp, 650 bp, 731 bp, 812 bp, 891 bp, 972 bp, 1070 bp, 1070 bp + 972 bp, 650 bp + 972 bp, 731 bp + 972 bp, 812 bp + 972 bp, and few unspecific bands. Conversely, certain studies have documented monomorphic *coa* gene profiles, such as Abdeen *et al.* (2015) [1] with 630 bp, suggesting strain-specific characteristics or limited genetic diversity within their isolates. Other researchers, including Nazir *et al.* (2017) [25] and Ibrahim *et al.* (2019) [16], observed dual amplicons (e.g., 910 and 730 bp; 680 and 790 bp), reinforcing the polymorphic nature of the *coa* gene.

In the present study, three isolates were found to be negative in the tube coagulase test (TCT) at the 5-hour mark, yet were positive for the *coa* gene via PCR. This discrepancy highlights the limitation of relying solely on phenotypic assays for coagulase detection, which may be influenced by expression levels, testing conditions, or protein stability. Therefore, molecular confirmation using *coa* gene PCR is essential to accurately classify MRSA isolates and avoid false-negative results from phenotypic assays.

Conclusion

This study underscores the importance of integrating phenotypic and molecular approaches for accurate identification of *Staphylococcus aureus*. While the tube coagulase test remains a useful diagnostic tool, its variability across plasma sources and incubation periods may lead to misinterpretation. PCR-based detection of the *coa* gene offers greater reliability and specificity, highlighting its value in confirming coagulase production. The observed genetic diversity in *coa* gene profiles further emphasizes the need for molecular typing to understand strain variation. Overall, combining traditional and molecular diagnostics enhances the accuracy of MRSA detection and epidemiological insights.

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